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(54) Title: BETA-SECRETASE ISOLATED FROM HUMAN 293 CELLS

(57) Abstract

Compositions comprising a protease isolated from human 293 cells or polypeptide fragments having about 85 % sequence homology with said protease are disclosed. The native protease is designated β-secretase and cleaves β-amyloid precursor protein on the amino-terminal side of the β-amyloid peptide. The β-secretase has an apparent MW from about 260-300 kD, when glycosylated and binds wheat germ agglutinin. A screening assay for identification of β-secretase inhibitors shows that said β-secretase is not inhibited by common inhibitors of serine, cysteine, aspartyl and metalloproteases.

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BETA-SECRETASE ISOLATED FROM HUMAN 293 CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the cleavage of β-amyloid precursor protein to produce β-amyloid peptide. More particularly, the present invention relates to isolated and purified compositions containing an enzyme responsible for such cleavage (β-secretase) and assays for identifying inhibitors of β-secretase.

Alzheimer's disease is characterized by the presence of numerous amyloid plaques and neurofibrillary tangles (highly insoluble protein aggregates) present in the brains of Alzheimer's disease patients, particularly in those regions involved with memory and cognition. β-amyloid peptide is a major constituent of amyloid plaque which is produced by cleavage of β-amyloid precursor protein. It is presently believed that a normal (non-pathogenic) processing of the β-amyloid precursor protein occurs via cleavage by a putative "α-secretase" which cleaves between amino acids 16 and 17 of the β-amyloid peptide region within the protein. It is further believed that pathogenic processing occurs in part via a putative "β-secretase" which cleaves at the amino-terminus of the β-amyloid peptide region within the precursor protein. Heretofore, however, the existence of β-secretase has not been confirmed.

The identification, isolation, and characterization of novel biological molecules having unique activities is generally useful. For example, novel enzymes can be used to catalyze reactions of a type associated with their class. In particular, novel proteases can be used to cleave proteins for a variety of purposes, and the availability of new proteases provides unique capabilities. In addition to such uses associated with enzymes in general, the identification,

isolation, and purification of the putative β-secretase enzyme would permit chemical modeling of a critical event in the pathology of Alzheimer's disease and would allow the screening of compounds to determine their ability to inhibit β-secretase activity.

For these reasons, it would be desirable to isolate, purify, and characterize the enzyme responsible for the pathogenic cleavage of β-amyloid precursor protein at the amino-terminus of the β-amyloid peptide region. In particular, it would be desirable to utilize such an enzyme (referred to hereinafter as β-secretase) in methods for screening candidate drugs for the ability to inhibit the activity of β-secretase in *in vitro* systems. It would be particularly desirable if such screening assays could be performed in a rapid format which would permit the screening of large numbers of test drugs in automated fashion.

2. Description of the Background Art

β-amyloid precursor protein is expressed in three differently-spliced forms of 695, 751, and 770 amino acids, and "normal" processing involves proteolytic cleavage at a site between residues Lys^{16} and Leu^{17} in the β-amyloid peptide. Kang et al. (1987) *Nature* 325:773-776. Soluble β-amyloid peptide which has been cleaved at the putative β-secretase site has also been found in the culture medium of non-diseased cells (Haass et al. (1992) *Nature* 359:322-325) and in CSF from healthy humans and animals (Seubert et al. (1992) *Nature* 359:325-327). The possible existence of the putative β-secretase is discussed in, for example, Selkoe, "Cell Biology of the Amyloid β-Protein and the Mechanism of Alzheimer's Disease," in *Annual Review of Cell Biology*, Spudich et al., eds., Annual Review, Inc., Palo Alto, California, vol. 10, 1994.

SUMMARY OF THE INVENTION

The present invention provides a novel β-secretase composition comprising an isolated and purified enzyme which cleaves β-amyloid precursor protein (APP) at the amino-

polypeptide as the β-secretase facilitates detection of cleavage by capture of the amino-terminal portion and labelling of the amino-terminal portion.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the elution profile of β-secretase purified from human 293 cells produced by gel exclusion chromatography on Superdex 200.

Fig. 2 is a similar elution profile shown for β-secretase purified from human brain.

Fig. 3 is an electrophoretic gel elution profile of β-secretase activity.

Fig. 4 is a schematic illustration of an APP-containing fusion peptide useful in performing the screening assays of the present invention, having a binding epitope derived from maltose-binding protein (MBP). An assay is run by exposing the fusion polypeptide to β-secretase which cleaves the 125 amino acid portion of APP (APP C-125) at the amino-terminus of the βAP. The MBP portion may then be captured, and the carboxy-terminus of the APP fragment which is exposed by cleavage with β-secretase may be identified with 192 antibody specific for said terminus. SW-192 antibody bound to a reporter is utilized, which antibody recognizes the carboxy-terminus of the Swedish mutation of APP.

Fig. 5 illustrates APP 638 which is a recombinantly expressed form of APP truncated after βAP (Aβ). APP 638 may be used in a β-secretase assay where the βAP peptide is cleaved and the carboxy-terminus of the amino-terminal fragment of APP 638 recognized by 192 antibody in either a Western blot or ELISA assay. The carboxy terminal βAP fragment can also be measured using a 3D6/266 assay.

Fig. 6 is the complete amino acid sequence of the Swedish mutation of the fusion polypeptide of maltose-binding protein and APP fragment utilized in the Experimental section hereinafter (SEQ ID No.:3).

Fig. 7 is a standard curve generated for the β-secretase ELISA described in detail in the Experimental section below.

Fig. 8 is an anionic exchange column elution profile obtained for deglycosylated β-secretase enzyme.

Fig. 9 is a Western blot of the deglycosylated β-secretase enzyme probed with 238B antisera.

Fig. 10 is an elution profile of the β-secretase enzyme from a cationic exchange column.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a novel protease which cleaves the β-amyloid precursor protein (APP) at the amino-terminus of the β-amyloid peptide (βAP) therein. It is believed that this protease is the putative β-secretase responsible for the pathogenic processing of APP to produce βAP in βAP-related conditions, such as Alzheimer's disease, Down's syndrome, HCHWA-D, and the like. Thus, the novel protease of the present invention will be referred to hereinafter as "β-secretase." The β-secretase of the present invention will be useful as a protease in *in vitro* and *in vivo* systems where proteases may generally find use. For example, β-secretase may be used to cleave or attempt to cleave proteins in order to characterize, process, modify, or otherwise react with the protein as a substrate. Thus, β-secretase will have general utility as a proteolytic chemical reagent in a wide variety of chemical reactions and systems. In addition, the β-secretase of the present invention will have a specific utility in the performance of screening assays to identify β-secretase inhibitors, *i.e.*, test compounds which are able to inhibit the proteolytic cleavage of APP in the presence of β-secretase. Such assays will be described in detail below.

As used herein, "β-amyloid precursor protein" (APP) refers to a polypeptide that is encoded by a gene of the same name localized in humans on the long arm of chromosome 21 and that includes a βAP region (defined below) within its carboxyl third. APP is a glycosylated, single-membrane-spanning protein expressed in a wide variety of cells in many mammalian tissues. Examples of specific isotypes of APP which are currently known to exist in humans are the 695-amino acid

polypeptide described by Kang et al. (1987) *Nature* 325:733-736, which is designated as the "normal" APP. A 751-amino acid polypeptide has been described by Ponte et al. (1988) *Nature* 331:525-527 and Tanzi et al. (1988) *Nature* 331:528-530. A 770-amino acid isotype of APP is described in Kitaguchi et al. (1988) *Nature* 331:530-532. A number of specific variants of APP have also been described having point mutations which can differ in both position and phenotype. A general review of such mutations is provided in Hardy (1992) *Nature Genet.* 1:233-234. A mutation of particular interest is designated the "Swedish" mutation where the normal Lys-Met residues at positions 595 and 596 are replaced by Asn-Leu. This mutation is located directly upstream of the normal β-secretase cleavage site of APP, which occurs between residues 596 and 597 of the 695 isotype.

As used herein, "β-amyloid peptide" (βAP) refers to the approximately 43 amino acid peptide which comprises residues 597-640 of the 695 amino acid isotype of APP. BAP is produced by processing of the APP including cleavage at both the amino-terminus and carboxy-terminus of the region. It is believed that the β-secretase of the present invention is responsible for cleavage of APP at the amino-terminus of βAP in normal and pathogenic processing of APP in human cells.

β-secretase has been characterized in a number of respects, as described in detail in the Experimental section below. β-secretase has an apparent molecular weight in the range from 260 kD to 300 kD, with a tentative molecular weight of 280 kD, as determined by gel exclusion chromatography in 0.2% hydrogenated Triton X-100. β-secretase will bind to wheat germ agglutinin but not to concanavalin A. It has been found to have a net negative charge at pH 4.75 and pH 5 (where it does not bind to a cationic exchange material) and a net negative charge at pH 7.5 (where it binds to an anion exchange material). The β-secretase of the present invention will cleave both wild-type (normal) and the Swedish mutation of APP at the putative β-secretase cleavage site on the immediate amino-terminal side of the βAP fragment, and has been found to have a higher proteolytic activity with respect to the Swedish

form of APP. Proteolytic activity appears to be at its peak at a pH from 5 to 5.5, with very low activity at pH 7.5 and above. β-secretase is resistant to many known protease inhibitors (see Table 1 in the Experimental section below). β-secretase appears to recognize only those polypeptide substrates which have retained a substantial number of residues upstream and downstream from the cleavage site (from either the wild-type, Swedish, or other mutated form) of APP, with an oligopeptide analog including 17 residues upstream and 16 residues downstream from the cleavage site (with a total of 33 amino acids) being resistant to cleavage by β-secretase.

The β-secretase of the present invention will be provided in an isolated and purified form. By "isolated and purified," it is meant that the β-secretase has been either (1) isolated and at least partially purified from a natural source, such as human brain tissue or human 293 cells (as described in detail in the Experimental section below) or (2) is produced recombinantly and synthetically. At present, as neither the amino acid sequence nor the nucleic acid sequence of the β-secretase gene have been determined, it is preferred that β-secretase be obtained from cellular sources using known protein purification techniques. Contaminating proteins may be removed from the β-secretase co-positions by specific techniques, including serial lectin chromatography on agarose-bound succinylated-wheat germ agglutinin (SWGA) and agarose-bound lentil lectin (LCA). These lectins, although partly binding β-secretase activity, preferentially bind other contaminating proteins in the purified fractions, and thus allow increased enrichment of the β-secretase activity. The β-secretase will be isolated and purified to an extent sufficient to increase the β-secretase activity in the resulting composition to a useful level. In particular, the β-secretase preparations of the present invention will have sufficient activity to cleave APP and APP-containing polypeptides as described in the Experimental section below. Preferably, the β-secretase compositions of the present invention will have an activity which is at least 10-fold greater than that of a solubilized but unenriched membrane

fraction from human 293 cells. More preferably, the compositions will have a β-secretase activity which is at least about 100-fold greater than that of the solubilized membrane fraction from human 293 cells. A specific method for determining β-secretase activity in units of "ng m^{-1} h^{-1}" is described in detail in the Experimental section below (see footnote 1 to Table 3).

In addition to compositions comprising a naturally-occurring β-secretase molecule, the present invention comprises compositions including polypeptides comprising fragments of β-secretase as well as polypeptides substantially homologous to the intact β-secretase or a portion thereof. The β-secretase polypeptides of the present invention will generally exhibit at least about 80% sequence homology with the naturally-occurring sequence of the full-length β-secretase or an active fragment thereof, *i.e.*, a fragment which displays APP cleavage activity. Usually, the β-secretase polypeptides will exhibit at least about 85% sequence homology, more usually at least about 95% sequence homology, and often 99% sequence homology or higher. The length of comparison sequences will generally be at least about 16 amino acid residues, usually at least about 20 residues, more usually at least about 24 residues, typically at least about 28 residues, and preferably more than about 35 residues.

The β-secretase polypeptides of the present invention may also be in the form of fusion polypeptides where an active fragment of the β-secretase molecule is joined to all or a portion of another protein. Fusions may be generated with heterologous proteins (for example, a reporter polypeptide, a binding polypeptide, or the like). Fusion polypeptides may be formed either recombinantly or by synthetic methods which are well-known in the art.

The β-secretase polypeptides of the present invention may also have amino acid residues which have been chemically modified by known techniques, such as phosphorylation, sulfonation, biotinylation or the addition of other moieties. In some embodiments, the modifications will

be useful for labelling reagents, purification targets, affinity ligands targeting, or the like.

The β-secretase polypeptides of the present invention may be used to prepare polyclonal and/or monoclonal antibodies using conventional techniques with the β-secretase as an immunogen. The intact β-secretase molecule, or fragments thereof, optionally coupled to a carrier molecule, may be injected into small vertebrates, with monoclonal antibodies being produced by well-known methods. See, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, New York, and Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2nd ed.) Academic Press, New York. Antibodies produced from β-secretase will be useful for performing conventional immunoassays to detect β-secretase in biological and other specimens.

The β-secretase compositions described above will be particularly useful for performing *in vitro* assays for detecting β-secretase inhibitors, where at least partially purified β-secretase is combined with the polypeptide substrate comprising the β-secretase cleavage site of APP in the presence of the test substrate. Conditions are maintained such that the β-secretase would cleave the polypeptide substrate into an amino-terminal fragment and a carboxy-terminal fragment in the absence of a substance which inhibits such cleavage. Cleavage of the polypeptide substrate in the presence of the test compound is compared with that in the absence of the test compound, and those test substances which provide significant inhibition of the cleavage activity (usually at least about 25% inhibition, more usually at least about 50% inhibition, preferably at least about 75% inhibition, and often at least about 90% inhibition or higher) are considered to be β-secretase inhibitors. Such β-secretase inhibitors may then be subjected to further *in vitro* and/or *in vivo* testing to determine if they inhibit the production of βAP in cellular and animal models. Suitable *in vivo* and *in vitro* tests are described in copending application Serial Nos.

07/965,972 and 07/831,722, the full disclosures of which are incorporated herein by reference.

Suitable substrate polypeptides will include a region of the APP molecule which is recognized and cleaved by β-secretase. Usually, the substrate polypeptide will include at least about 15 amino acid residues, preferably at the least about 25 amino acids, on the amino-terminal side of the cleavage site (located between amino acids 596 and 597 in the 695-amino acid isomer) and at least about 28 amino acids, preferably at least about 42 amino acids, on the carboxy-terminal side of the cleavage site. The cleavage site will typically comprise the Met-Asp or the Leu-Asp cleavage site characteristic of the wild-type and Swedish forms of βAPP. An intact APP molecule will be suitable as the polypeptide including both wild-type and mutant forms of APP, particularly including the Swedish mutation of APP. Use of fusion substrate polypeptides is often preferred, where an affinity region can be fused to the β-secretase cleavage site of APP, producing a molecule whose cleavage can be conveniently monitored in solid phase test systems.

The screening assays of β-secretase and suitable substrate polypeptide are conveniently performed using "sandwich" assays where the amino-terminal or the carboxy-terminal fragment produced by cleavage is captured on a solid phase. The captured fragment may then be detected using an antibody specific for the end of the fragment exposed by β-secretase cleavage. In an exemplary embodiment described in detail in the Experimental section below, the polypeptide substrate is a fusion polypeptide combining maltose-binding protein and a 125-amino acid carboxy-terminal fragment of APP. The assay uses anti-maltose-binding protein antibody to capture the amino-terminal cleavage product, where the carboxy-terminus of the cleavage product is detected by an antibody specific thereto. An exemplary antibody is 192 antibody or SW-192 antibody, described in more detail in copending application 08/143,697, filed on October 27, 1993, the full disclosure of which is incorporated herein by reference. The binding of the antibody to the cleaved fusion

polypeptide is detected using conventional labelling systems, such as horseradish peroxidase or other detectable enzyme labels, which are bound to the antibody directly (covalently), or indirectly through intermediate linking substances, such as biotin and avidin.

The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL

Purification and Characterization of β-Secretase

Frozen tissue (293 cell paste or human brain) was cut into pieces and combined with five volumes of homogenization buffer (20 mM Hepes, pH 7.5, 0.25 M sucrose, 2 mM EDTA). The suspension was homogenized using a blender and centrifuged at 1000 x g (10 min, 4°C) to produce a post-nuclear supernatant which was saved on ice. The pellets were resuspended in fresh homogenizing buffer at the original volume, and the centrifugation step was repeated. The second supernatant was combined with the first one, and the supernatant pool ("PNS") was centrifuged at 16,000 x g for 30 min at 4°C. The supernatants were discarded and the pellets, labelled "P2," were either used immediately for enzyme purification or frozen at -40°C for later use.

The pellets were suspended in extraction buffer (20 mM MES, pH 6.0, 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml E64, 1 μg/ml pepstatin, 0.2 mM PMSF) at the original volume. After vortex-mixing, the extraction was completed by agitating the tubes at 4°C for a period of one hour. The mixtures were centrifuged as above at 16,000 x g, and the supernatants were pooled. The pH of the extract was adjusted to 7.5 by adding ~1% (v/v) of 1 M Tris base (not neutralized).

The neutralized extract was loaded onto a wheat germ agglutinin-agarose (WGA-agarose) column pre-equilibrated with 10 column volumes of 20 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA, at 4°C. One milliliter of the agarose resin was used for every 4 g of original tissue used. The WGA-column was washed with 10 column volumes of the

equilibration buffer, and then eluted as follows. Three-quarter column volumes of 1 M N-acetylglucosamine in 20 mM Tris, pH 7.5, 0.5% Triton X-100, 2 mM EDTA were passed through the column after which the flow was stopped for fifteen minutes. An additional five column volumes of the 1 M N-acetylglucosamine elution buffer were then used to elute the column, followed by five column volumes of 10% chitin hydrolysate in 20 mM Tris, pH 7.5, 0.5% Triton X-100, 2 mM EDTA. All of the above eluates were combined (pooled WGA-eluate).

The pooled WGA-eluate was diluted 1:4 with 20 mM NaOAc, pH 5.0, 0.5% Triton X-100, 2 mM EDTA. The pH of the diluted solution was adjusted to 5.0 by adding a few drops of glacial acetic acid while monitoring the pH. This "SP load" was passed through a 5-ml Pharmacia HiTrap SP-column equilibrated with 20 mM NaOAc, pH 5.0, 0.5% Triton X-100, 2 mM EDTA, at 4 ml/min at 4°C. β-Secretase activity was present in the flow-through fraction, which was neutralized by adding enough 1 M Tris (not neutralized) to bring the pH up to 7.5. The enzyme solution was then loaded onto a 1-ml Pharmacia HiTrap Q-column equilibrated with approximately 10 column volumes of 20 mM Tris, pH 7.5, 0.2% hydrogenated Triton X-100, 2 mM EDTA, at 1.5 ml/min at 4°C. The column was washed with 10 column volumes of 20 mM Tris, pH 7.5, 0.2% hydrogenated Triton X-100, 50 mM NaCl, 2 mM EDTA. Protein was eluted using a linear gradient from 50 mM TO 350 Mm NaCl over 30 minutes at a flow-rate of 1 ml/min at 4°C. The protein concentrations in the HiQ fractions were measured using a BioRad colorimetric protein assay, and the β-secretase activity was measured using the MBP-C125 cleavage assay at pH 5.5. The fractions in the ascending portion of the protein peak have the highest specific activity and were pooled for further purification of the enzyme.

The pooled fractions from the HiTrap Q were then applied to a column of concanavalin A-agarose (10% v/v of pool) equilibrated with 10 column volumes of 20 mM Tris, pH 7.5, 0.2% hydrogenated Triton X-100, 150 mM NaCl, 2 mM EDTA. The Con A flow-through was then loaded onto a

Superdex 200 (26/60) gel exclusion chromatography column, which was eluted with phosphate buffered saline, pH 7.4, 0.2% hydrogenated Triton X-100, 2 mM EDTA, at 1 ml/min, collecting 3 min/fraction. Fractions containing β-secretase activity were identified using the MBP-C125 cleavage assay. The apparent molecular weight of the β-secretase activity eluting from the Superdex column was estimated from the peak elution volume (relative to that of standard proteins) to be 280,000 ± 9800 (average of two runs for 293 cells, and two runs for human brain).

Results from a large-scale preparation of the enzyme from human brain tissue is shown in Table 1 below.

Table 1

Step	Activity ng/ml/h	Protein μg/ml	Sp. Act.[1] ng/ml/h/μg protein	Fold Purfn.
Solubilized membrane extr.	2700	350	7.7	1
HiQ Elution pool	80000	210	380.9	49.5
Con A Flow-Thru	80000	100	800	103.8
Superdex peak fraction	57000	< 5	> 11400	> 1480.5

[1] Specific activity of the purified β-secretase was measured as follows. MBP C125-SW (described below) was combined at approximately 0.7 μg/ml in 100 mM sodium acetate, pH 5.5, with 0.3% Triton X-100. The amount of product generated was measured by the β-secretase assay, also described below. Specific activity was then calculated as:

$$Sp.Act. = \frac{(Product\ conc.\ ng/ml)(Dilution\ factor)(Incubation\ vol.\ \mu l)}{(Enzyme\ sol.\ vol.\ \mu l)(Incubation\ time\ h.)(Enzyme\ conc.\ \mu g/ml)}$$

The Sp. Act. is thus expressed as ng of protein produced per μg of β-secretase per hour.

In order to analyze for protein, a portion (360 μl) of each fraction was concentrated by acetone precipitation,

then analyzed by SDS-PAGE and Coomassie blue staining on 10% Tris-Glycine gels. Fig. 1 shows the protein pattern of the Superdex 200 (26/60) elution profile for β-secretase purified from 293 cells, and Fig. 2 shows the elution profile for β-secretase purified from human brain. A number of protein bands are visible in the fractions with β-secretase activity. In order to further characterize the β-secretase, an aliquot of substantially purified β-secretase was electrophoresed under non-denaturing conditions into a 6% separating-4% stacking native gel system, according to the method of Laemmli (*Nature*, (1970) 227:680), except that the SDS is replaced by 0.2% hydrogenated triton X-100. Following electrophoresis, the gel is soaked for ~30 minutes in 0.1 M sodium acetate, pH 5.5. The lane of electrophoresed protein (7 cm long x 1 cm wide x 1 mm thick) is then cut into ~2.5 mm pieces using a clean razor blade, sequentially from the top of the stacker to the bottom of the separating gel. Each of these slices is combined with 60 μl water and 10 μl 1 M sodium acetate in a microcentrifuge tube, then homogenized using a Kontes Deltaware motorized pellet pestle microhomogenizer. MBP-C125 SW is added to the desired concentration (0.5-0.7 μg/ml), and the mixture incubated overnight. The samples are then diluted with Specimen Diluent (defined below) 50-fold, and analyzed by the β-secretase activity ELISA assay. The results are shown in Fig. 3.

Glycosylation of β-secretase has been investigated using various immobilized lectins, and ability of substantially purified β-secretase activity to bind to them was determined. Table 2 summarizes this data. A "-" sign signifies less than 20% binding, "+" between 24-40% binding, "++" between 50-75% binding, and "+++" > 75% binding.

Table 2

Lectin	β-Secretase Binding
jequirity bean (APA)	+
jack bean (con A)	+
scotch broom (CSA)	+
jimson weed (DSA)	+
coral tree (ECA)	-
grifornia simplicifolia I	-
grifornia simplicifolia II	-
Jacalin (AIA)	+
lentil (LCA)	+
horseshoe crab (LPA)	-
tomato (LEL)	+
maackia (MAA)	+
peanut (PNA)	+
pokeweed (POA)	-
castor bean (RCA1)	-
potato (STL)	-
wheat germ - succinylated (SWGA)	+
China gourd (TKA)	+
stinging nettle (UDA)	+
gorse (UEAI)	-
gorse (UEAII)	-
hairy vetch (VVL)	-
wheat germ (WGA)	+++

Only a single lectin (WGA) bound β-secretase activity quantitatively, out of the many tested. Partial binding of the activity (25-40%) to a number of other lectins probably indicates heterogeneous glycosylation.

β-secretase purified as described was assayed in the presence of a number of common protease inhibitors as follows. Enzyme solution was mixed with the inhibitor, as described below in the β-Secretase Inhibitor Assay section, and assayed for activity remaining as a percentage of a control solution incubated under otherwise identical conditions. IC_{50} values, if any, were determined as the concentration of inhibitor which resulted in 50% inhibition of the control activity. The results are set forth in Table 3.

Table 3

Inhibitor	Max Conc	IC50
SERINE PROTEASES		
aminoethylbenzene-sulfonyl fluoride	0.8 mM	NI
chymostatin	0.2 mM	NI
3,4-dichloroisocoumarin	0.5 mM	< 25% inh.
diisopropylfluoro-phosphate	2 mM	NI
elastatinal	0.2 mM	NI
phenylmethylsulfonyl-fluoride	1.0 mM	NI
CYSTEINE PROTEASES		
E-64	0.14 mM	NI
N-ethylmaleimide	10 mM	NI
iodoacetamide	10 mM	NI
METALLOPROTEASES		
EDTA	2 Mm	NI
phosphoramidon	10 mM	NI
o-phenanthroline		7 mM
m-phenanthroline		7 mM
ASPARTYL PROTEASES		
pepstatin	25 μM	NI
diazoacetylnorleucyl-methyl ester		> 5 mM
DIVALENT METAL IONS		
Cu		2 mM
Zn		3 mM
Hg		< 10% inh
Ca		NI
Mg		NI

These results indicate that β-secretase activity is not inhibited by common inhibitors of serine, cysteine, aspartyl, and metalloproteases. Although o-phenanthroline inhibits poorly, m-phenanthroline, which is not a metal chelator, also does so, suggesting that this weak inhibition is unrelated to the metal-chelating properties of o-phenanthroline.

A potentially contaminating protein was detected in the β-secretase preparations. Antisera were raised against a synthetic peptide sequence (NH_2-CGGEAKGAEDAPDADTA-$CONH_2$ [SEQ ID NO. 4]) corresponding to a 14-amino-acid sequence in the predicted carboxy-terminal end of this new polypeptide with a CGG linker at the amino-terminal end of the peptide. This antisera (238B) detected a family of polypeptides with an indistinguishable pattern as seen previously with other antisera. However, 238B showed greater sensitivity on Western blots, and can be used to detect these polypeptides in the purified enzyme preparations.

The further purification of the β-secretase enzymatic activity was achieved by developing a protocol to substantially deglycosylate the glycoprotein(s) present in the purified enzyme preparation, while maintaining enzymatic activity. The removal of the Asn-linked carbohydrate residues was effected by treatment with the enzyme PNGase F. Following this, the deglycosylated enzyme preparation was subjected to sequential anion-exchange and cation-exchange chromatography.

Enzymatic deglycosylation was performed as follows. Flow-through from LCA-agarose lectin chromatography (~15 ml) which contained the β-secretase activity, was diluted 1:4 and bound to ~50:1 of Pharmacia DEAE-Sepharose Fast Flow beads, batchwise. The DEAE-Sepharose beads were then collected by low-speed centrifugation, washed once with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.2% R-TX100 (MiniQ Buffer A), followed by a elution in five volumes of Buffer A containing 450 mM NaCl. This step allowed for the concentration of the enzymatic activity into a small volume prior to the addition of the deglycosylating enzyme.

The eluate from the DEAE-Sepharose beads (~250:1) was then treated with 1/20 (v/v) of recombinant PNGase F (Glyko, 2.5 U/ml) for 4-5 d at 37°C, in the presence of 3 mM β-mercaptoethanol. This treatment removed Asn-linked carbohydrate chains from glycoproteins.

Anion-exchange chromatography was then performed as follows. The deglycosylated enzyme sample was diluted 1:10 in MiniQ Buffer A, and loaded on to a MiniQ PC3.213 (Pharmacia) anion-exchange column equilibrated with this buffer. The column was washed with 10 volumes of 30 mM NaCl in MiniQ Buffer A, followed by elution with a linear gradient to 500 mM NaCl in Buffer A over 20 min, at a flow rate of 0.4 ml/min. Fractions (0.4 ml) were analyzed for both β-secretase activity utilizing the MBP-C125Sw cleavage assay (Fig 8), as well as immunoreactivity for the previously detected polypeptide using antisera 238B analyzed by Western blotting after Tris-Tricine PAGE on 10-20% acrylamide Novex gels (Fig 9). Fractions (7-10) corresponding to the peak of enzymatic activity contained partially resolved forms of the polypeptides recognized by the 238B antisera, ranging in apparent MW from between approx 48 kDa to approx 148 kDa.

Cation-exchange chromatography was used to further purify the enzymatic activity. The peak activity fractions from the MiniQ elution were diluted 1:5 in SP Buffer A (20 mM NaOAc, pH 4.75, 2 mM EDTA, 0.2% R-TX100) in order to alter the pH as well as lower the ionic strength prior to cation exchange chromatography. The diluted enzyme pool was mixed with 50:1 of a 50% slurry of Pharmacia SP-Sepharose® beads. Following overnight mixing at 4°C, the beads were collected by low speed centrifugation, and the supernatant ("SP Flow-Through") was saved for further analysis. The SP-Sepharose beads were eluted stepwise with 100 mM NaCl increments in SP Buffer A, up to 600 mM NaCl. These fractions (E1-E6, ~0.45 ml each), along with the SP Flow-Through, and an aliquot of the SP Load, were analyzed for both β-secretase activity as well as immunoreactivity for the previously identified polypeptide. The results are shown in Fig 10. All of the immunoreactive bands bind to the SP-Sepharose, since they are quantitatively

depleted in the SP-Flow Through, and elute in fractions E2-E4. In contrast, the β-secretase enzymatic activity quantitatively flows through (95% recovered in the SP Flow-Through), and thus is separated from the contaminating polypeptides.

β-Secretase Inhibitor Assay

β-secretase assays utilizing the SW-192 antibody, which recognizes the free ...Val-Asn-Leu-COOH terminal sequence uncovered proteolytic cleavage immediately amino-terminal of the βAP sequence, were performed. Two recombinantly-expressed proteins (Figs. 4 and 5) have been used as substrates for β-secretase. The preferred substrate (Fig. 4) was expressed in *E. coli* as a fusion protein of the last 125 aa of APP fused to the carboxy-terminal end of maltose-binding protein, using commercially available vectors from New England Biolabs. The β-cleavage site was thus 26 amino acids downstream of the start of the C-125 region.

Recombinant proteins were generated with both the wild-type APP sequence (MBP-C125 WT) at the cleavage site (..Val-Lys-Met-Asp-Ala..) [SEQ ID No.:1] or the "Swedish" double mutation (MBP-C125 SW) (..Val-Asn-Leu-Asp-Ala..) [SEQ ID No.:2]. The entire sequence of the recombinant protein with the Swedish sequence is given in Fig. 6 [SEQ ID No.:3]. As shown in Fig. 4, cleavage of the intact MBP-fusion protein results in the generation of a truncated amino-terminal fragment, with the new SW-192 Ab-positive epitope uncovered at the carboxy terminus. This amino-terminal fragment can be recognized on Western blots with the same Ab, or, quantitatively, using an anti-MBP capture-biotinylated SW-192 reporter sandwich format, as shown in Fig. 4.

Anti-MBP polyclonal antibodies were raised in rabbits (Josman Labs, Berkeley) by immunization with purified recombinantly expressed MBP (New England Biolabs). Antisera were affinity purified on a column of immobilized MBP. MBP-C125 SW and WT substrates were expressed in *E. coli*, then purified using affinity binding to and elution from immobilized amylose-agarose matrix (New England Biolabs). The

purified substrates were stored frozen at -40°C in 3 M guaninide-HCl and 1% Triton X-100, @ ~0.7 mg/ml.

Microtiter 96-well plates were coated with purified anti-MBP antibody (@ 5-10 μg/ml), followed by blocking with human serum albumin. β-secretase solution (1-10 μl) is mixed with substrate (0.5 μl) in a final volume of 50 μl, with a final buffer composition of 20 mM sodium acetate, pH 5.5, 0.03% Triton X-100, in individual wells of 96-well microtiter plates, and incubated at 37°C for 2 h. Samples were then diluted 5-fold with Specimen Diluent (0.2 g/l sodium phosphate monobasic, 2.15 g/l sodium phosphate dibasic, 0.5 g/l sodium azide, 8.5 g/l sodium chloride, 0.05% Triton X-405, 6 g/l BSA), further diluted 5-10 fold into Specimen Diluent on anti-MBP coated plates, and incubated for 1 h. Biotinylated SW192 antibodies were used as the reporter. SW192 polyclonal antibodies were biotinylated using NHS-biotin (Pierce), following the manufacturer's instruction. Usually, the biotinylated antibodies were used at about 240 ng/ml, the exact concentration varying with the lot of antibodies used. Following incubation of the plates with the reporter, the ELISA was developed using streptavidin-labeled alkaline phosphatase (Boeringer-Mannheim) and 4-methyl-umbelliferyl phosphate as fluorescent substrate. Plates were read in a Cytofluor 2350 Fluorescent Measurement System. Recombinantly generated MBP-26SW (product analog) was used as a standard to generate a standard curve (Fig. 7), which allowed the conversion of fluorescent units into amount of product generated.

This assay protocol was used to screen for inhibitor structures, using "libraries" of compounds assembled onto 96-well microtiter plates. Compounds were added, in a final concentration of 20 μg/ml in 2% DMSO, in the assay format described above, and the extent of product generated compared with control (2% DMSO only) β-secretase incubations, to calculate "% inhibition." "Hits" were defined as compounds which result in >35% inhibition of enzyme activity at test concentration. Using this system, 70 "hits" were identified out of the first 6336 compounds tested, a "hit" rate of ~1.1%.

Thus, the assay has been shown to be capable of distinguishing "non-inhibitors" (the majority of compounds) from "inhibitors."

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Athena Neurosciences, Inc.

(ii) TITLE OF INVENTION: Beta-Secretase

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Townsend and Townsend and Crew LLP
(B) STREET: Two Embarcadero Center, 8th Floor
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94111-3834

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE: herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Heslin, James M.
(B) REGISTRATION NUMBER: 29,541
(C) REFERENCE/DOCKET NUMBER: 015270-002230PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-326-2400
(B) TELEFAX: 415-326-2422

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Lys Met Asp Ala
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Asn Leu Asp Ala
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1521 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAAAACTG AAGAAGGTAA ACTGGTAATC TGGATTAACG GCGATAAAGG CTATAACGGT 60

CTCGCTGAAG TCGGTAAGAA ATTCGAGAAA GATACCGGAA TTAAAGTCAC CGTTGAGCAT 120

CCGGATAAAC TGGAAGAGAA ATTCCCACAG GTTGCGGCAA CTGGCGATGG CCCTGACATT 180

ATCTTCTGGG CACACGACCG CTTTGGTGGC TACGCTCAAT CTGGCCTGTT GGCTGAAATC 240

ACCCCGGACA AAGCGTTCCA GGACAAGCTG TATCCGTTTA CCTGGGATGC CGTACGTTAC 300

AACGGCAAGC TGATTGCTTA CCCGATCGCT GTTGAAGCGT TATCGCTGAT TTATAACAAA 360

GATCTGCTGC CGAACCCGCC AAAAACCTGG GAAGAGATCC CGGCGCTGGA TAAAGAACTG 420

AAAGCGAAAG GTAAGAGCGC GCTGATGTTC AACCTGCAAG AACCGTACTT CACCTGGCCG 480

CTGATTGCTG CTGACGGGGG TTATGCGTTC AAGTATGAAA ACGGCAAGTA CGACATTAAA 540

GACGTGGGCG TGGATAACGC TGGCGCGAAA GCGGGTCTGA CCTTCCTGGT TGACCTGATT 600

AAAAACAAAC ACATGAATGC AGACACCGAT TACTCCATCG CAGAAGCTGC CTTTAATAAA 660

GGCGAAACAG CGATGACCAT CAACGGCCCG TGGGCATGGT CCAACATCGA CACCAGCAAA 720

GTGAATTATG GTGTAACGGT ACTGCCGACC TTCAAGGGTC AACCATCCAA ACCGTTCGTT 780

GGCGTGCTGA GCGCAGGTAT TAACGCCGCC AGTCCGAACA AAGAGCTGGC GAAAGAGTTC 840

CTCGAAAACT ATCTGCTGAC TGATGAAGGT CTGGAAGCGG TTAATAAAGA CAAACCGCTG 900

GGTGCCGTAG CGCTGAAGTC TTACGAGGAA GAGTTGGCGA AAGATCCACG TATTGCCGCC 960

ACCATGGAAA ACGCCCAGAA AGGTGAAATC ATGCCGAACA TCCCGCAGAT GTCCGCTTTC 1020

TGGTATGCCG TGCGTACTGC GGTGATCAAC GCCGCCAGCG GTCGTCAGAC TGTCGATGAA 1080

GCCCTGAAAG ACGCGCAGAC TAATTCGAGC TCGGTACCCG GCCGGGGATC CATCGAGGGT 1140

AGGGCCGACC GAGGACTGAC CACTCGACCA GGTTCTGGGT TGACAAATAT CAAGACGGAG 1200

GAGATCTCTG AAGTGAATCT GGATGCAGAA TTCCGACATG ACTCAGGATA TGAAGTTCAT 1260

CATCAAAAAT TGGTGTTCTT TGCAGAAGAT GTGGGTTCAA ACAAAGGTGC AATCATTGGA 1320

CTCATGGTGG GCGGTGTTGT CATAGCGACA GTGATCGTCA TCACCTTGGT GATGCTGAAG 1380

AAGAAACAGT ACACATCCAT TCATCATGGT GTGGTGGAGG TTGACGCCGC TGTCACCCCA 1440

GAGGAGCGCC ACCTGTCCAA GATGCAGCAG AACGGCTACG AAAATCCAAC CTACAAGTTC 1500

TTTGAGCAGA TGCAGAACTA G 1521

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys Gly Gly Glu Ala Lys Gly Ala Glu Asp Ala Pro Asp Ala Asp Thr
1 5 10 15

Ala

WHAT IS CLAIMED IS:

1. A composition of matter comprising an isolated and purified enzyme which cleaves β-amyloid precursor protein at the β-amyloid peptide cleavage location.

2. A composition of matter comprising β-secretase, wherein the composition has a β-secretase activity which is at least five-fold greater than that of a solubilized but unenriched membrane fraction from human 293 cells.

3. A composition of matter as in claim 2, wherein the β-secretase activity is at least about 100-fold greater than that of a solubilized but unenriched membrane fraction from human 293 cells.

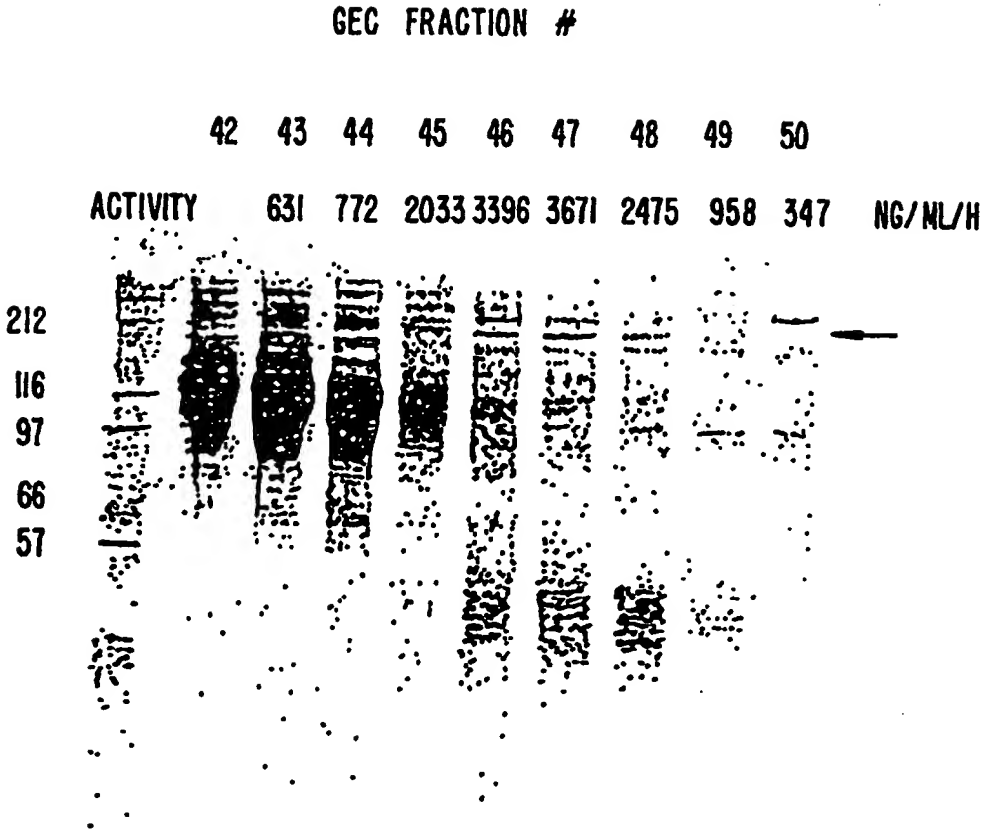
4. A composition of matter as in claim 1 comprising at least 10% by weight of the enzyme, wherein the enzyme is further characterized by:

(a) an apparent molecular weight when glyosylated in the range from 260 kD to 300 kD when measured by gel exclusion chromatography in hydrogenerated Triton X-100;

(b) a gel pattern as in Fig. 1 or 2;

(c) a net negative charge at pH 5 and a net negative charge at pH 7.5; and

(d) binds to wheat germ agglutinin with partial binding to other lectins as set forth in Table 2.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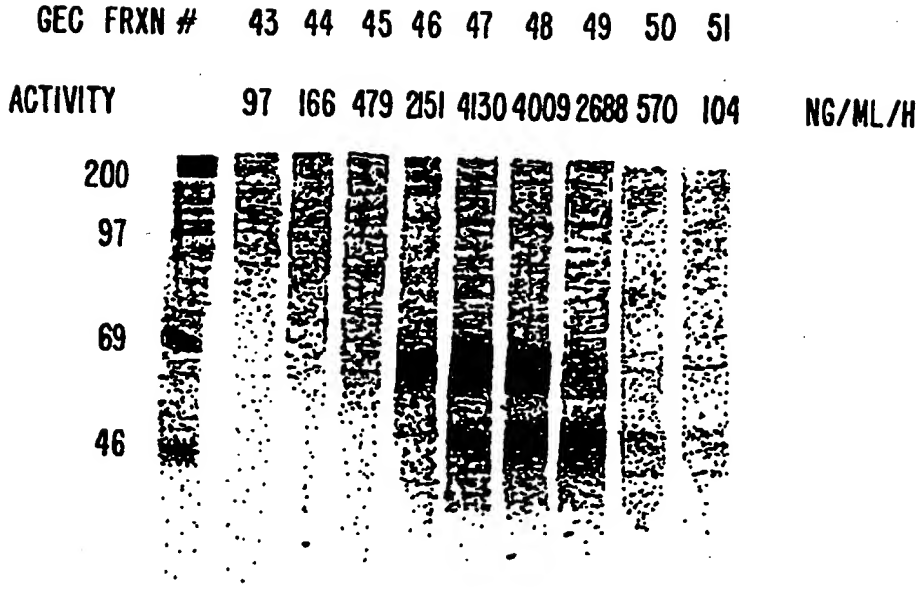
ACTIVITY 631 772 2033 3396 3671 2475 958 347 NG/ML/H
212
116
97
66
57

FIG. 1.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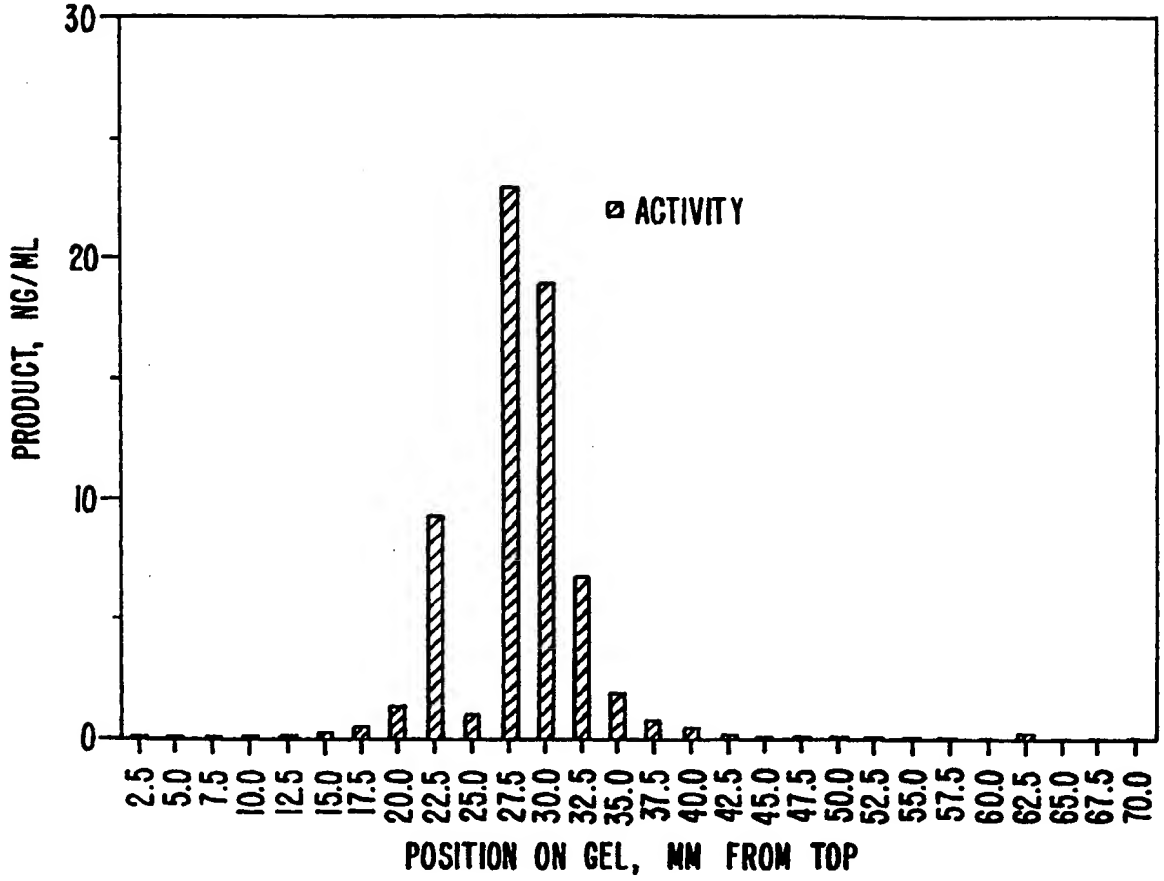
200
97
69
46

FIG. 2.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10
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17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
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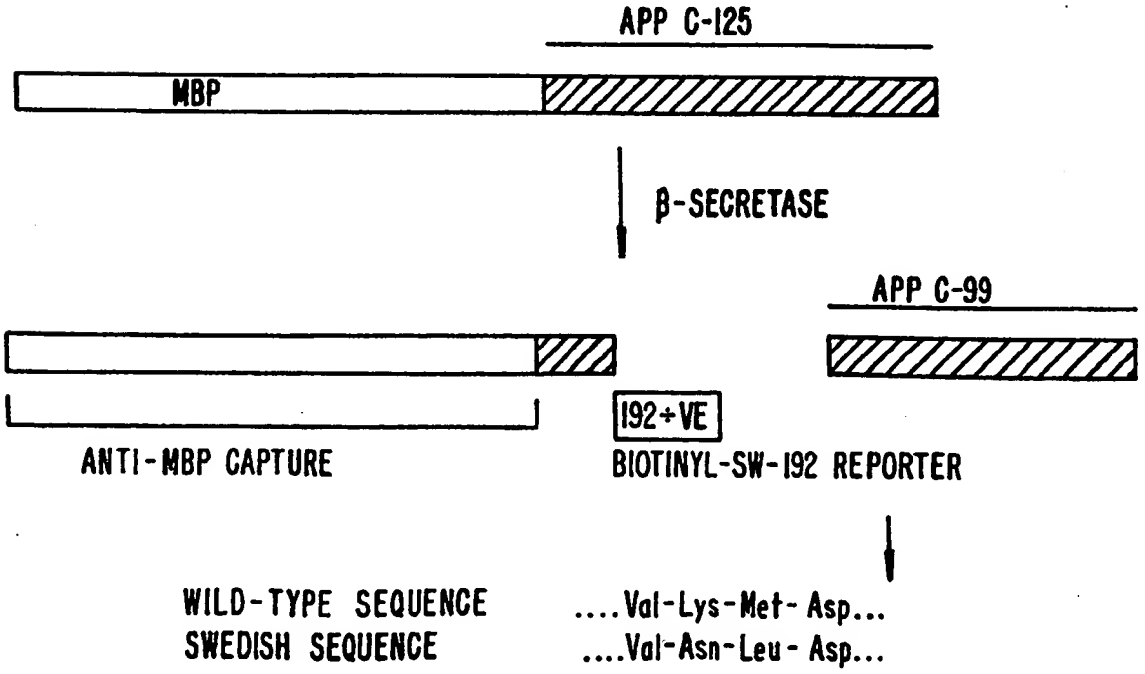
62.5
65.0
67.5
70.0
POSITION ON GEL, MM FROM TOP

FIG. 3.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MBP
β-SECRETASE
APP C-99
192+VE
ANTI-MBP CAPTURE
BIOTINYL-SW-192 REPORTER
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SWEDISH SEQUENCE ....Val-Asn-Leu-Asp...

FIG. 4.

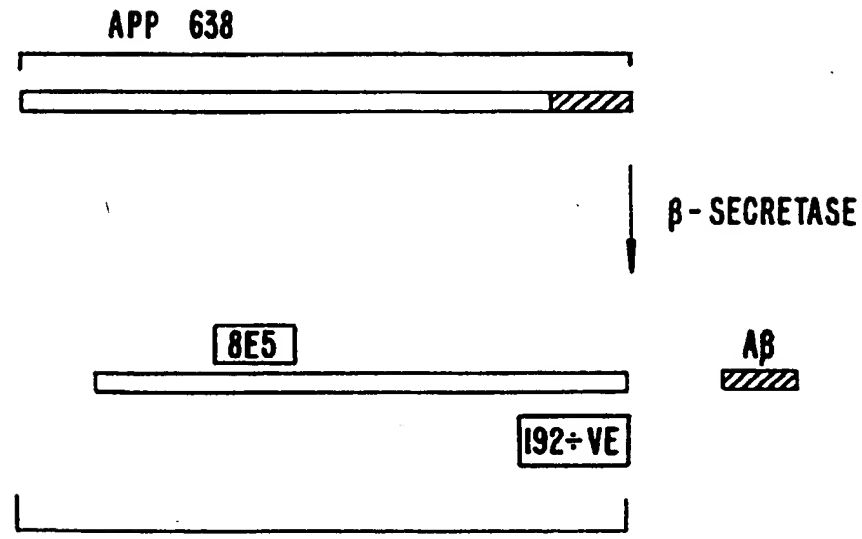
APP 638
β-SECRETASE
8E5
Aβ
192÷VE
DETECTED BY: 1. SW 192 WESTERN BLOT
2. 8E5-192 ELISA
WILD-TYPE SEQUENCE
....Val - Lys - Met - Asp...
SWEDISH SEQUENCE
....Val - Asn - Leu - Asp...

FIG. 5.

```
  1 ATGAAAACTGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAG
  1 MetLysThrGluGluGlyLysLeuValIleTrpIleAsnGlyAspLysGlyTyrAsnGlyLeuAlaGluValGlyLys

 79 AAATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCG
 27 LysPheGluLysAspThrGlyIleLysValThrValGluHisProAspLysLeuGluGluLysPheProGlnValAla

157 GCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCT
 53 AlaThrGlyAspGlyProAspIleIlePheTrpAlaHisAspArgPheGlyGlyTyrAlaGlnSerGlyLeuLeuAla

235 GAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGCAAGCTG
 79 GluIleThrProAspLysAlaPheGlnAspLysLeuTyrProPheThrTrpAspAlaValArgTyrAsnGlyLysLeu

313 ATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGG
105 IleAlaTyrProIleAlaValGluAlaLeuSerLeuIleTyrAsnLysAspLeuLeuProAsnProProLysThrTrp

391 GAAGAGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAAGAACCGTAC
131 GluGluIleProAlaLeuAspLysGluLeuLysAlaLysGlyLysSerAlaLeuMetPheAsnLeuGlnGluProTyr

469 TTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAAAACGGCAAGTACGACATTAAAGACGTG
157 PheThrTrpProLeuIleAlaAlaAspGlyGlyTyrAlaPheLysTyrGluAsnGlyLysTyrAspIleLysAspVal

547 GGCGTGGATAACGCTGGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGAC
183 GlyValAspAsnAlaGlyAlaLysAlaGlyLeuThrPheLeuValAspLeuIleLysAsnLysHisMetAsnAlaAsp

625 ACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCC
209 ThrAspTyrSerIleAlaGluAlaAlaPheAsnLysGlyGluThrAlaMetThrIleAsnGlyProTrpAlaTrpSer

703 AACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCATCCAAACCGTTCGTT
235 AsnIleAspThrSerLysValAsnTyrGlyValThrValLeuProThrPheLysGlyGlnProSerLysProPheVal
```

FIG. 6-1.

```
 731 GGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAACAAAGAGCTGGCGAAAGAGTTCCTCGAAAACTATCTGCTG
 261 GlyValLeuSerAlaGlyIleAsnAlaAlaSerProAsnLysGluLeuAlaLysGluPheLeuGluAsnTyrLeuLeu

 859 ACTGATGAAGGTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTG
 287 ThrAspGluGlyLeuGluAlaValAsnLysAspLysProLeuGlyAlaValAlaLeuLysSerTyrGluGluGluLeu

 937 GCGAAAGATCCACGTATTGCCGCCACCATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCC
 313 AlaLysAspProArgIleAlaAlaThrMetGluAsnAlaGlnLysGlyGluIleMetProAsnIleProGlnMetSer

1015 GCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTGAAAGAC
 339 AlaPheTrpTyrAlaValArgThrAlaValIleAsnAlaAlaSerGlyArgGlnThrValAspGluAlaLeuLysAsp

1093 GCGCAGACTAATTCGAGCTCGGTACCCGGCCGGGGATCCATCGAGGGTAGGGCCGACCGAGGACTGACCACTCGACCA
 365 AlaGlnThrAsnSerSerSerValProGlyArgGlySerIleGluGlyArgAlaAspArgGlyLeuThrThrArgPro

1171 GGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGACATGACTCAGGA
 391 GlySerGlyLeuThrAsnIleLysThrGluGluIleSerGluValAsnLeuAspAlaGluPheArgHisAspSerGly

1249 TATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATG
 417 TyrGluValHisHisGlnLysLeuValPhePheAlaGluAspValGlySerAsnLysGlyAlaIleIleGlyLeuMet

1327 GTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCAT
 443 ValGlyGlyValValIleAlaThrValIleValIleThrLeuValMetLeuLysLysLysGlnTyrThrSerIleHis

1405 CATGGTGTGGTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTACGAA
 469 HisGlyValValGluValAspAlaAlaValThrProGluGluArgHisLeuSerLysMetGlnGlnAsnGlyTyrGlu

1483 AATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAG
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FIG. 6-2.

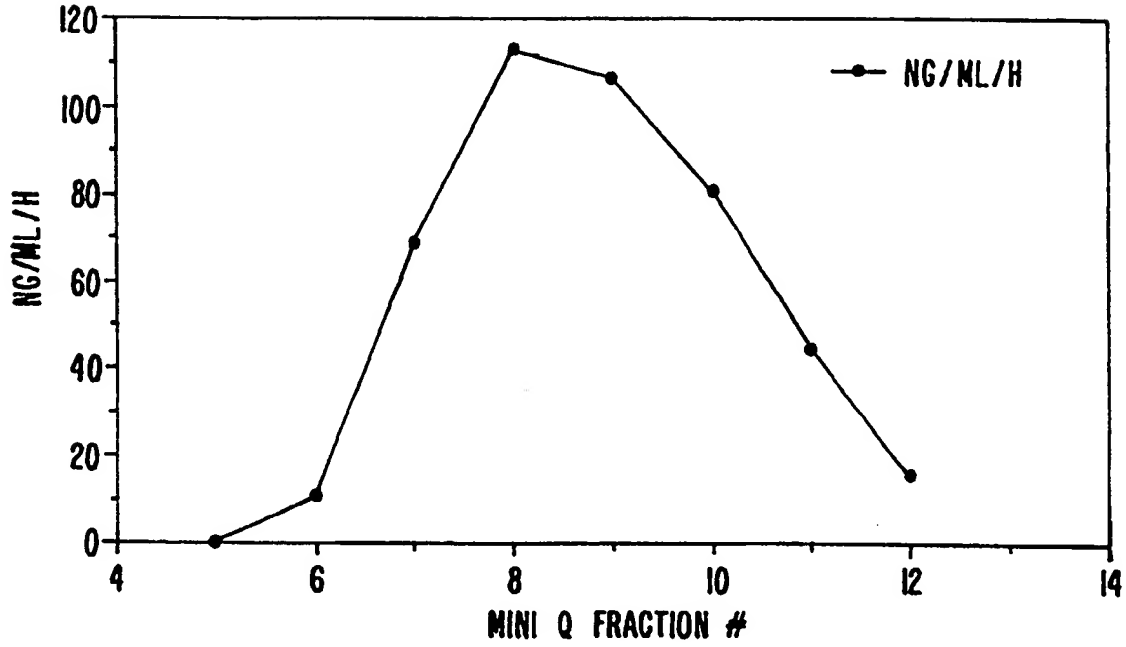

120
100
80
60
40
20
0
NG/ML/H
NG/ML/H
4
6
8
10
12
14
MINI Q FRACTION #

FIG. 8.

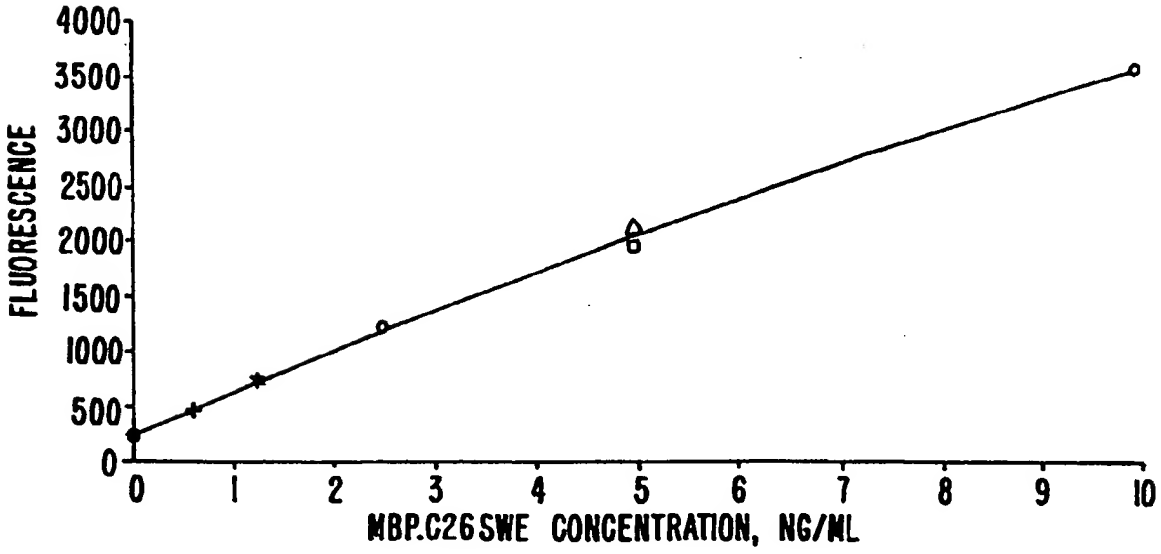
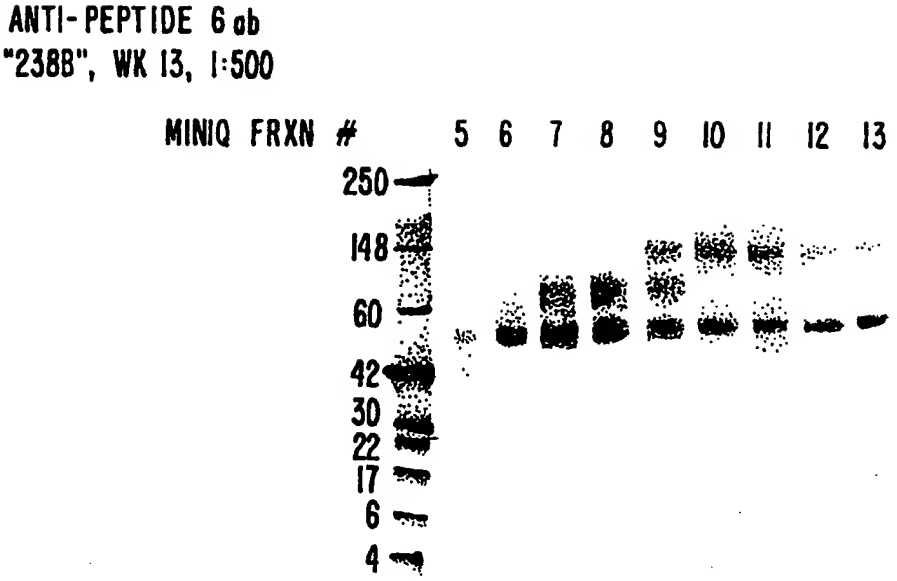

4000
3500
3000
2500
2000
1500
1000
500
0
FLUORESCENCE
0
1
2
3
4
5
6
7
8
9
10
MBP.C26SWE CONCENTRATION, NG/ML

FIG. 7.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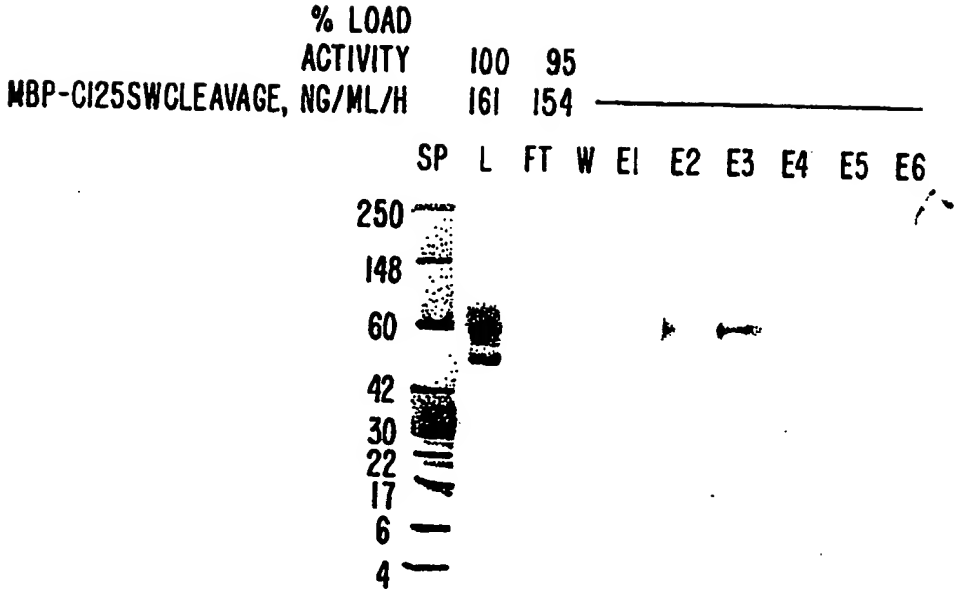
"238B", WK 13, 1:500
MINIQ FRXN # 5 6 7 8 9 10 11 12 13
250
148
60
42
30
22
17
6
4

FIG. 9.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ACTIVITY 100 95
MBP-CI25SWCLEAVAGE, NG/ML/H 161 154
SP L FT W E1 E2 E3 E4 E5 E6
250
148
60
42
30
22
17
6
4

FIG. 10.

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US96/19549

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 9/48; C12P 21/04
US CL :435/212, 70.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/219, 212, 195, 363, 366, 368, 70.1, 70.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/13904 A1 (CEPHALON, INC.) 19 September 1991, pages 46-47, 51-52 and Figures 4, 9 and 10.	1-3
X	GLENNER et al. Alzheimer's and Parkinson's Diseases: Recent Developments. Advances in Behavioral Biology. New York: Plenum Press. 1995, Vol. 44, pages 127-130, especially page 129, third and seventh paragraphs.	1
X	US 5,424,205 A (DOVEY et al.) 13 June 1995, columns 13-14.	1-3
X	TAGAWA et al. Alzheimer's disease amyloid β-clipping enzyme (APP secretase): Identification, purification, and characterization of the enzyme. Biochem. Biophys. Res. Comm. 1991, Vol. 177, No. 1, pages 377-387, especially pages 379-380.	1

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search 23 APRIL 1997	Date of mailing of the international search report 13 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN HANLEY JAB for Telephone No. (703) 308-0916

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PCT/US96/19549

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NELSON et al. Identification of a chymotrypsin-like mast cell protease in rat brain capable of generating the N-terminus of the Alzheimer amyloid β-protein. Journal of Neurochem. 1993, Vol. 61, No. 2, pages 567-577, especially pages 571-572.	1
A	SAMBAMURTI et al. Evidence for intracellular cleavage of the Alzheimer's amyloid precursor in PC12 cells. Journal of Neurosci. Res. 1992, Vol. 33, pages 319-329, entire document.	1-4

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PCT/US96/19549

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, BIOSIS, WPIDS, MEDLINE, REGISTRY
search terms: beta-secretase, beta-amyloid precursor protein, Alzheimer's, human 293 cell, beta-protein amyloidogenase, protease